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A biosensor based on magnetoresistance technology

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Abstract: We are developing a biosensor that will measure, at the level of single molecules, the forces that bind DNA–DNA, antibody–antigen, or ligand–receptor pairs together. The Bead Array Counter (BARC) will use these interaction forces to hold magnetic microbeads to a solid substrate. Microfabricated magnetoresistive transducers on the substrate will indicate whether or not the beads are removed when pulled by magnetic forces. By adapting magnetoresistive computer memory technology, it may be possible to fabricate millions of transducers on a chip and detect or screen thousands of analytes. The multi-analyte capability of this portable sensor would be ideal for on-site testing, while the potential to directly guage intermolecular interaction strengths suggests drug discovery applications.

Keywords: intermolecular forces, giant magnetoresistance, magnetic beads.

INTRODUCTION

Measuring recognition forces with the atomic force microscope

Over the past decade, various methods of directly measuring DNA–DNA, antibody–antigen, and ligand–receptor interaction forces have been devised to aid research into biomolecular recognition. These methods all involve the controlled application of force to two biomolecule-covered surfaces that are brought into contact and then separated. Methods that use large contact areas, such as the surface forces apparatus (Wong *et al.*, 1997) and radial or linear fluid flow bead detachment assays (Kuo and Lauffenburger, 1993; Pierres *et al.*, 1996), have provided some of the most quantitative and useful information to date. However, methods such as micropipet-mounted membrane capsules (Evans *et al.*, 1995) and atomic force microscopy (AFM) (Lee *et al.*, 1994a; Ludwig *et al.*, 1997) may ultimately provide more detailed and unambiguous records of molecular binding and unbinding because of their ability to measure forces between individual molecular pairs.

The atomic force microscope has, for example, been used to measure the interaction force between two molecules of DNA (Lee *et al.*, 1994; Florin *et al.*, 1995). In one such experiment (Fig. 1) (Lee *et al.*, 1994), single-stranded DNA in the form of polycytosine 20mers was covalently attached to the tip of a cantilever-beam force transducer. Polycytosine was also attached to an immobile substrate. Free strands of polyinosine averaging 160 bases in length were introduced. When the tip and substrate were brought together, a polyinosine strand would sometimes bind to polycytosine on both the tip and the substrate, thus bridging these two surfaces. The tip and substrate were then pulled apart. In the case of Fig. 1, the cantilever did not sense any force until some slack in the DNA was taken up, at which point tension on the DNA began pulling the cantilever down (negative force). When this force reached –600 pN, the DNA–DNA bond at either the tip or substrate broke, therefore eliminating the force on the cantilever. The appearance of only one negative peak or break point indicates that only one polyinosine strand bridged the tip and sample. Because no adhesion force is observed when polyinosine is not present, in a sense the AFM has detected the presence of a single molecule.

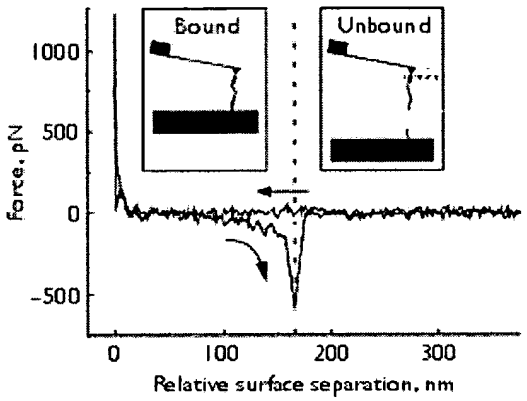

Fig. 1. Interaction force between two complementary strands of DNA as measured by AFM. "Relative surface separation" is the distance between the tip and sample relative to the position at which 1000 pN of force is reached. Measurements are recorded both as the tip and sample are brought together (thin trace) and as they are separated (thick trace).

AFM has similarly been used to measure forces between other biomolecules, most significantly (for the present work) antibody–antigen pairs (Hinterdorfer *et al.*, 1996; Dammer *et al.*, 1996; Allen *et al.*, 1997). However, such recognition force measurements remain an exploratory, basic research activity. It is our goal to detect and characterize biomolecular interactions on a routine basis for applications such as drug screening and on-site multianalyte testing. To achieve this goal, we are developing a method of measuring recognition forces, molecule-by-molecule, with magnetic microbeads. This method is derived from AFM but is much better suited to our intended applications.

Although it is ideal for basic research, the AFM is not a practical biosensor. The chemically-active portion of an AFM consists of only a few molecules at the apex of a sharp tip. Analyte molecules must be individually located and probed, so an AFM needs at least 15 minutes to acquire a significant number of observations (~100) for a single analyte. During this time the tip contacts the substrate numerous times and may become contaminated or otherwise inactivated, in which case the tip must be washed or replaced. Although tip contamination is not a significant problem for basic research applications, it could become significant if measurements on hundreds of potentially impure "real-world" samples were attempted. Furthermore, the AFM is a fairly complex instrument that requires a low-vibration environment, extensive user training, and continual monitoring by the user.

In a variant of the usual AFM experiment, Hoh et al. (1994) have used magnetic force microscopy (MFM) to indirectly apply forces to biomolecular bonds. Streptavidin-biotin bonds were allowed to form between derivatized magnetic beads and a substrate. A magnetic AFM tip (i.e., an MFM tip) was then

repeatedly scanned a diminishing distance above the substrate, thus exerting increasing force on the beads. The deflection of the cantilever, recorded in a sequence of MFM images, indicated the amount of force applied. Eventually the bonds holding the beads to the substrate broke, at which point the beads jumped onto the MFM tip and disappeared from the MFM images. This "magnetic jump" method eliminates artifacts seen in AFM experiments when the motion of the tip is not perpendicular to the plane of the sample or parallel to the ligand-receptor axis (Stuart and Hlady, 1995). However, like AFM, this method detects only one molecule at a time.

THE BEAD ARRAY COUNTER CONCEPT

Magnetic bead assays

We are developing a method that uses magnetic microbeads to detect and characterize many individual biomolecular interaction events simultaneously. Our method derives from the experiment depicted in Fig. 1, but has similarities to the magnetic jump method. Rather than bridging a substrate and an AFM tip, the target DNA (or antigen) molecule bridges the substrate and a magnetic microbead. By allowing an aqueous suspension of derivatized magnetic beads to settle onto the substrate, thousands of beads are allowed to bind to the substrate in this way, each ideally by a single target molecule. An external magnet then generates force on all of the beads, and the number of beads that remain on the substrate is measured optically or magnetically.

An important feature of this bead assay is that force is exerted perpendicular to the substrate. If beads experience forces parallel to the substrate, a fulcrum is created at the bead–substrate contact point, and the forces on the intermolecular bonds are magnified by a levering action much like that of a claw hammer (Chang and Hammer, 1996). Given that substrates and beads are not perfectly smooth, the lever geometry and therefore the degree of magnification can be difficult to control.

The application of controlled magnetic forces allows us to remove beads from the substrate based on their binding affinity. For example, we can separate specifically- from nonspecifically-bound beads. If nonspecific adhesion is minimized with antifouling surface treatments, a force of ~1 pN per bead applied for 5–10 seconds removes 99±1% of nonspecifically-adhering beads from a substrate lacking antibody or DNA probes (unpublished results). This level of force does not disturb beads bound via intermolecular recognition bonds.

The magnetic bead assay can be thought of as a binding assay that uses magnetic rather than radioactive or colorimetric label. Others have previously described the use of magnetic label and its potential as a simple and sensitive biosensor detection technique (Kotitz *et al.*, 1997; Kriz *et al.*, 1996; Kriz *et al.*, 1998). In our assay we have also attempted to replicate the ability of AFM to detect single target molecules, but with a technique that can be used on thousands of molecules simultaneously. The assay can therefore be incorporated into a biosensor having the unique ability to make accurate measurements of intermolecular forces.

The optimum application for such a biosensor depends in large part on the instrumentation used to detect the beads. Optical microscopy is perhaps the simplest way to count substrate-bound beads (Fig. 2). This method is used in our laboratory to develop methods for minimizing nonspecific adhesion; in the future it may be used for basic research into biomolecular recognition forces. Alternatively, the beads can be counted with a micromechanical force transducer that measures the total magnetic force exerted by the particles. We are developing this method as a high-sensitivity immunosensor for on-site detection of viruses, bacteria, and toxins (Baselt *et al.*, 1997). In this article, we describe a magnetoresistive detector that will support large arrays of molecular probes.

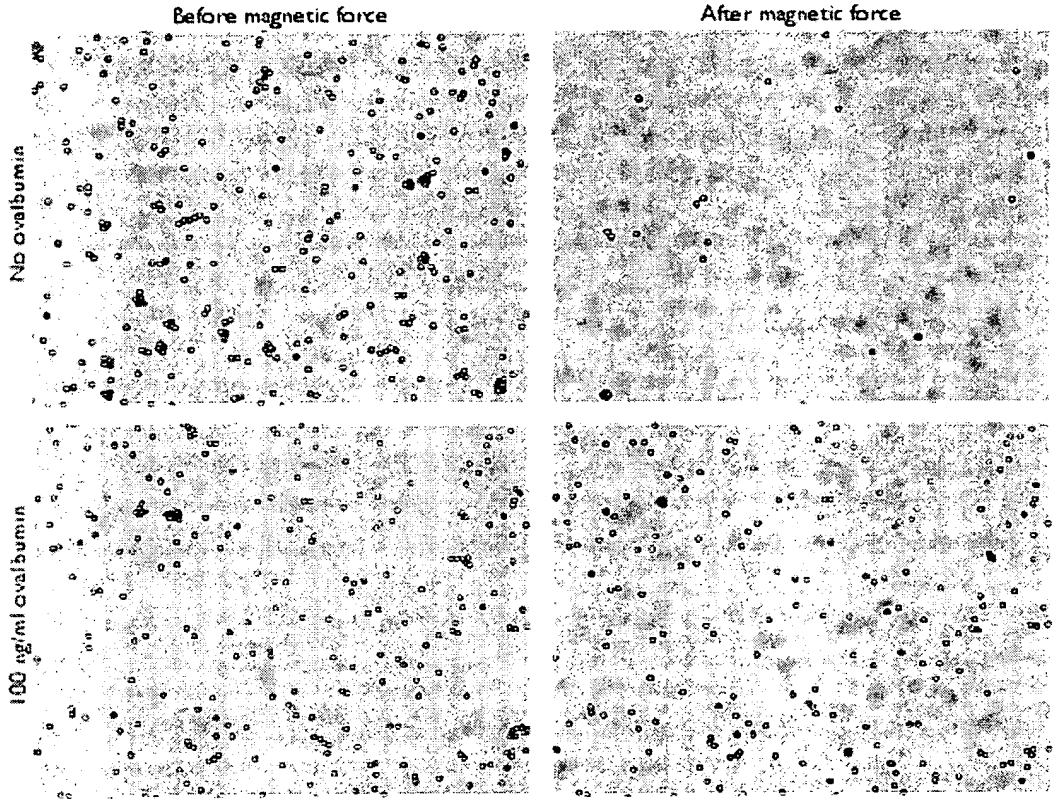

Fig. 2. Magnetic bead immunoassay for ovalbumin performed in a polystyrene microtiter well and observed by optical microscopy. When no ovalbumin is present, a 1 pN magnetic force applied for 5 s removes most of the Dynabeads M-280 that settle within the 250 x 200 μm field of view. There is some nonspecific adhesion of beads. When ovalbumin is present, a greater number of beads adhere to the surface and are not affected by the magnetic field. The number of adhering beads is roughly proportional to the logarithm of the ovalbumin concentration. Goat antirabbit (which were immobilized on the Dynabeads), goat antiovalbumin (on the substrate), and rabbit antiovalbumin (in solution) antibodies courtesy of J. Hager-Aldrich and T. O'Brien, Naval Medical Research Institute.

Principle of BARC

Recent developments in magnetoresistive materials have made it possible to photopattern highly-sensitive, micrometer-scale magnetic field sensors. Magnetoresistive materials are typically thin-film metal multilayers, the resistance of which changes in response to magnetic fields. Several fundamentally-different varieties have been described, including anisotropic magnetoresistive (AMR) and giant magnetoresistive (GMR) materials (see for example Baibich *et al.*, 1988). Magnetoresistive sensors are used commercially for reading magnetic tapes or disks, for hand-held magnetic field sensors, and for position transducers.

Using magnetoresistive materials, we can also produce a microfabricated detector to count magnetic beads. Such a detector can be embedded in the assay substrate and would detect the beads in its own immediate vicinity. The primary advantage of this approach over optical or micromechanical detection is that thousands of detectors can be fabricated on a single chip measuring about 1 cm on a side. Similar chips are expected to be commercially available as nonvolatile computer memories (magnetoresistive

RAM or MRAM) (Daughton, 1992) within three years.

We therefore envision the magnetoresistive-detection biosensor as a ~1000 analyte detector array — the *bead array counter* or BARC. However, it would be difficult to produce such large numbers of antibodies. Arrays containing thousands of DNA probes, on the other hand, can be synthesized and are even commercially available (O'Donnell-Maloney and Little, 1996). Therefore we are initially developing BARC as a DNA hybridization assay rather than an immunoassay. In particular, our program focuses on detection of multiple pathogens by PCR of blood samples (Long *et al.*, 1993) followed by hybridization to the BARC chip.

Nonetheless, we do hope to develop BARC as an immunoassay in the future, since its ability to measure binding affinities is likely to be of greatest use when applied to antibody-antigen or ligand-receptor pairs. Because separating DNA strands requires an order of magnitude more force [0.049–0.244 nN for antibody-antigen pairs (Allen *et al.*, 1997 and Hinterdorfer *et al.*, 1996, respectively) vs. 1.52 nN for 20 bp DNA (Lee *et al.*, 1994)] DNA binding force measurements may not even be possible with the present setup. In the BARC hybridization assay, the role of magnetic forces therefore will be limited to separating specific from nonspecific interactions.

A BARC assay might work as follows: microfabricated magnetoresistive elements (Fig. 3) are fabricated on a "detector chip." Such elements are typically coated with silicon nitride to prevent corrosion. A layer of silicon oxide can be added to enable covalent immobilization of DNA probes by any technique applicable to glass or silica surfaces, such as the aminosilane method of Chrisey *et al.* (1996). For each DNA sequence to be detected, an oligomer probe complementary to part of the sequence is synthesized and immobilized to a unique portion of the detector chip. The user injects biotinylated PCR product into the BARC instrument, where it flows through a disposable but multiuse liquid cell that contains the chip. The PCR product (i.e., the analyte) is hybridized to the complementary probes. Streptavidin-conjugated magnetic microbeads are then introduced and bind the biotin present on the analyte, thereby becoming immobilized to specific portions of the chip.

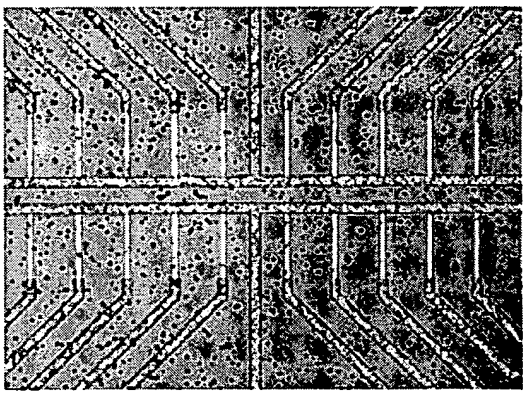

Fig. 3. Micrograph showing Dynabeads M-280 allowed to settle onto a prototype array of 80 x 5 μm GMR elements. To perform an assay, the microfabricated magnetoresistive sensing elements will be coated with DNA probes. A DNA hybridization assay will then be performed in which one magnetic bead binds to the chip for each DNA–DNA hybridization event that occurs. The number of magnetic beads that bind over each GMR element can be determined by measuring the resistance of that element. Beads that do not bind over sensing elements are ignored.

The result of this hybridization assay is that magnetic particles adhere to magnetoresistive sensing elements by DNA–DNA interactions (in series with streptavidin-biotin interactions). After using magnetic force to test the strength of the bonds and remove weakly-adhering beads, the detector chip counts the number of remaining beads. This count indicates the concentration of analyte DNA in the sample. Because beads that adhere between sensing elements are not counted and have no effect on the assay, the space between the elements is wasted. We may eventually be able to make high-density sensor arrays with little or no wasted space.

It is possible that more than one analyte molecule could connect a bead to the detector. However, this should not occur with any significant frequency unless the detector is saturated, in which case the analyte concentration should be reduced by diluting the sample.

Magnetic particles

A number of companies offer magnetic microbeads intended for biochemical separations, but also suitable for the assays that we are developing. The magnetic component of these particles is invariably iron oxide, usually in the form of maghemite (γ-Fe_2O_3). Typically, nanometer-sized particles of iron oxide are dispersed in, layered onto, or coated with a polymer or silica matrix to form beads about 1 μm in diameter. Nanometer-sized particles of iron oxide are only magnetic in the presence of a magnetic field; the particles immediately demagnetize when the field is removed, so the beads do not attract each other and agglomerate.

Iron oxide is the favored magnetic component because of its stability. However, since it is a ferrimagnetic (as opposed to ferromagnetic) material, it is not highly magnetic. Furthermore, commercial magnetic beads generally contain only 10–60% iron oxide. As a result, it is very difficult to exert more than ~10 pN of force per bead. Taking a force spectrum that encompasses the expected antibody–antigen unbinding force of 100 pN would require fabricating highly-magnetic beads. In our experience to date, however, such beads tend to agglomerate due to magnetic domain fringe fields or inadvertent magnetization. Another approach would be to use micrometer-scale magnets, as in the approach of Hoh et al. (1994). Small magnets can generate very large field gradients; however, since they only affect a small area they must be scanned over the sample. To date our efforts have focused on simply separating specifically- from nonspecifically-bound beads, which can be achieved with ~1 pN of force.

The experiments described in this article were performed with Dynabeads M-280 (Dynal, Inc., Lake Success, NY), which are 2.8 μm diameter polystyrene beads containing dispersed maghemite. Dynabeads are unique in that they have a highly uniform diameter. However, Dynabeads M-280 are not uniformly magnetic, [1] and their magnetic susceptibility is low when compared with other commercial magnetic beads (Fig. 4). Given a detection field of 8.0 kA/m, we calculate that these beads generate a maximum field of 0.3 mT within our GMR sensors (as will be illustrated in Fig. 7 below).

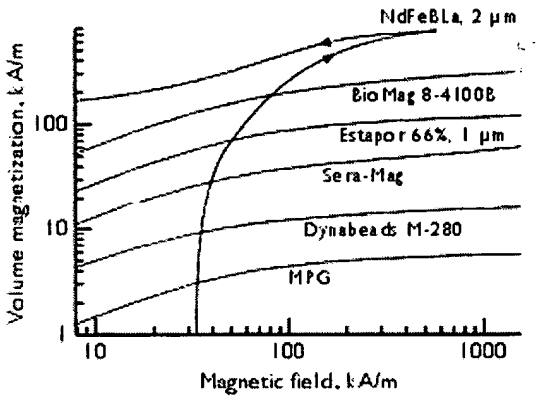

Fig. 4. Room-temperature volume magnetization loops for 1) custom-made NdFeBLa magnetic particles [2.00±1.10 µm diameter as measured by scanning electron microscopy, gas-atomized and aerodynamically size-classified, supplied by N. Koon, Naval Research Laboratory (NRL)]; 2) BioMag® Particulate Supports 8-4100B (nominally 1 µm nonspherical iron oxide particles from PerSeptive Diagnostics; 3) Estapor® Superparamagnetic Microspheres M0009400AN (1 µm nominal, 0.35±0.15 µm measured, 66% iron oxide, Bangs Laboratories); 4) Sera-Mag™ Magnetic Microparticles (0.85 µm diameter, 40% iron oxide, Seradyn, Inc.) 5) Dynabeads M-280 (2.8±0.2 µm diameter, 12% iron oxide, Dynal, Inc.); 6) MPG® Long Chain Alkylamine (nominal 5 µm diameter, 15% iron oxide, CPG, Inc.). Loop 1 from vibrating sample magnetometry (VSM) data acquired by Paul Dorsey, NRL; 2–6 from SQUID data acquired by Valerie Browning, NRL.

Magnetoresistive detection method

We have designed several prototype GMR sensor arrays (Fig. 5) in order to demonstrate magnetoresistive detection of magnetic particles. The prototype arrays were fabricated by Nonvolatile Electronics, Inc. (NVE; Eden Prairie, MN), and contain magnetoresistive strips fabricated from 0.1 µm thick GMR sandwich films (Dieny *et al.*, 1991) of proprietary composition. The resistance of the GMR material decreases by about 4% in response to a positive or negative field of 4 kA/m (Fig. 6). Electrical contact to the sensors is made via 1 µm thick aluminum leads. The entire chip is coated with 1 µm of silicon nitride and can be operated in salt solution.

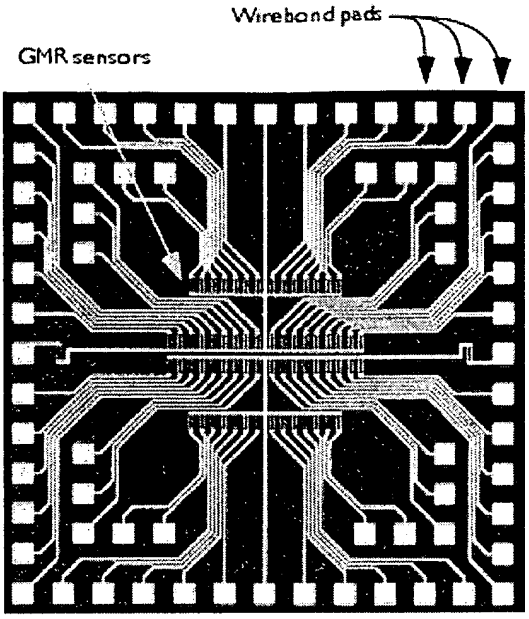

Fig. 5. Photograph of a prototype array of 64 magnetoresistive sensors. This particular array is composed of 80 x 20 μm GMR sensors. The GMR material is an NVE-proprietary "sandwich" film chosen for its high sensitivity to small magnetic fields.

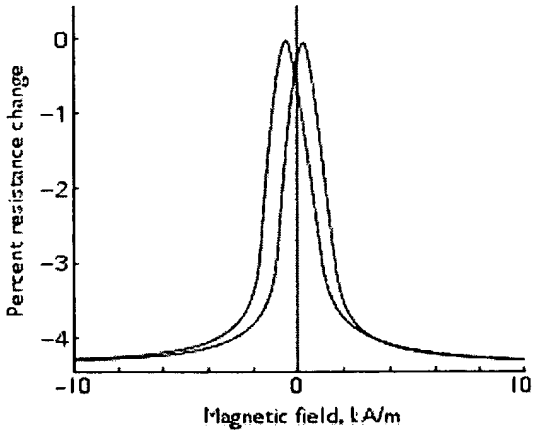

Fig. 6. Response characteristic of a 10 μm wide GMR test strip (25 mA current; measured by NVE, Inc.).

If the magnetoresistive sensors lie in the **XY** plane and current flows through them in the **X** direction, the sensors detect only the **X** component of the magnetic field. Therefore, to detect a superparamagnetic bead resting on a GMR strip, a magnetic field was externally generated in the **Z** direction, causing the bead to produce a magnetic field with a detectable **X** component (Fig. 7). The ≤0.3 mT magnetic induction produced by a Dynabead M-280 could thus be detected without being overwhelmed by the 10 mT induction of the magnetizing field.[2] However, since the magnetizing field was not homogeneous (and therefore not exactly perpendicular to the GMR sensors over the entire sensor array) the sensors exhibited an offset of 0.1–1 mT that varied with their position in the field. This offset was measured in the absence of magnetic beads and subtracted from subsequent measurements.

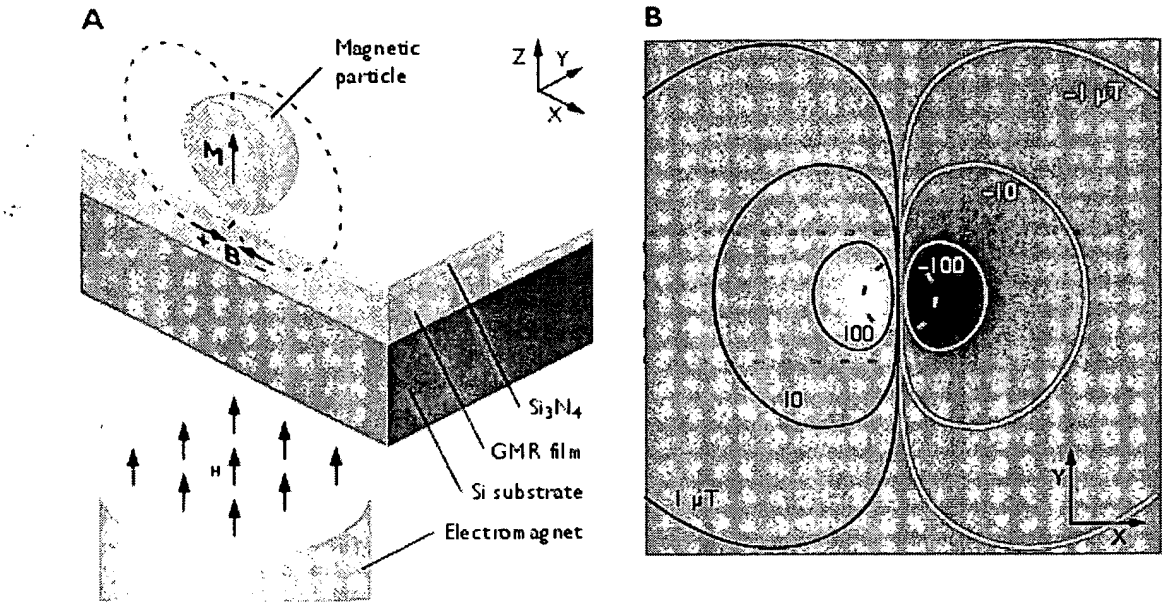

Fig. 7. A) Cross-section of a GMR sensor, illustrating the method used to detect superparamagnetic beads. A magnetizing field **H** magnetizes the bead, which produces regions of positive and negative magnetic induction **B** in the plane of the underlying GMR film. Because the film is only sensitive to the **X** component of external magnetic fields, the magnetizing field does not affect the GMR resistance. B) Calculated **X** component of the magnetic induction produced by a Dynabead M-280 in the plane of the GMR sensor when the magnetizing field **H**=4 kA/m and the bead is 1 μm above the sensor. The area shown measures 20 x 20 μm. The dotted circle indicates the position and size of the Dynabead, while the dotted rectangle indicates the position and size of a 20 x 5 μm GMR sensor. Current would flow through the sensor in the **X** direction.

To obtain an optimal signal-to-noise ratio, a "reference" GMR sensor identical to the main "signal" sensor was also used. The two sensors comprised half of a Wheatstone bridge (Fig. 8; the four-point bridge usually associated with GMR measurements was not used because it would have resulted in a prohibitively large number of leads). A constant bias voltage was chosen such that 2.5 mA of current passed through each GMR sensor for each micrometer of sensor width. The magnetizing field, provided by a standard commercial electromagnet (Magnet Sales and Mfg. Co., Culver City, CA) was modulated at 200 Hz, 4 kA/m RMS to enable lock-in detection.

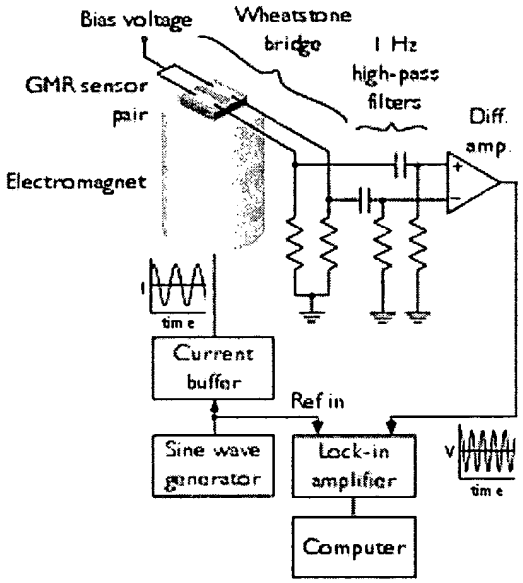

Fig. 8. Electronics used to detect magnetic beads. Two GMR elements (one signal and one reference) are incorporated into a Wheatstone bridge. The magnetizing-field electromagnet is modulated at 200 Hz and the resulting 400 Hz signal from the bridge detected with a lock-in amplifier. Highpass filters eliminate the need to manually balance the resistance of the signal and reference.

The signal-to-noise ratios resulting when one Dynabead M-280 is placed on various GMR sensors are shown in Fig. 9. It is readily apparent that electronic sensitivity increases with decreasing sensor surface area. As discussed below, however, chemical sensitivity increases with active area, i.e. the area in which DNA hybridization events can occur and be detected. Since magnetic beads can only be detected when they bind on top of a sensor, the active area is the total surface area of all sensors devoted to a particular analyte. Thus, the optimum BARC array would consist of a large number of small sensors.

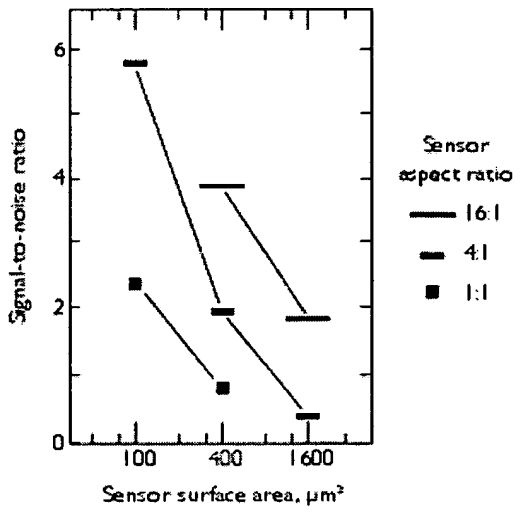

Fig. 9. Measured signal-to-noise ratio of seven different GMR sensors in response to one average Dynabead M-280. Using micromanipulation, a Dynabead was placed sequentially on two 20 x 5 μm sensors. For each sensor the average signal over a period of 102.4 s was measured as described in the text (noise is defined as the standard deviation of lock-in detector output over the same period; lock-in time constant was 1 s). The same bead was then placed on a sensor of a different size and/or aspect ratio, and the signal relative to that produced by the 20 x 5 μm sensors was determined. This entire process was repeated with four beads for each point on the plot. The signal from an *average* Dynabead was determined by testing the 20 x 5 μm sensors with 13 beads. The other data points were calculated based on the absolute signal of the 20 x 5 μm sensors and the six relative signal measurements.

Multi-analyte potential of BARC

Ultimately, the number of analytes that a BARC chip can accommodate will depend on the total active area available and the amount of area required per analyte. The total active area of our prototype arrays is limited because each element has a separate connection to the off-chip circuitry. Achieving the full

multi-analyte potential of BARC will ultimately require using on-chip switching circuitry to multiplex thousands or millions of elements to a single off-chip connection. Arrays containing 256,000 or more magnetoresistive elements are already being developed in the form of MRAM and could in principle be adapted for BARC — sensor design criteria such as access time, sensitivity, and density are less demanding for BARC than for MRAM. When using such an array, each probe species would be immobilized to a region containing perhaps several thousand sensors. The minimum size of this region will be determined by mass transport and bead density limitations.

If the active area per probe is too small, mass transport limitations could potentially impact assay sensitivity. The number of analyte molecules N that come into contact with the active area can be estimated as (Cussler, 1994):

$$N = kAt(C - C_0)$$

where k is the mass transport coefficient (~0.001 m/s), A is the active area, t is the amount of time allotted for hybridization, C is the concentration in molecules/m^3 of the analyte molecule in the solution applied to the sensor chip, and C_0 is the concentration at the sensor surface, initially zero. Assuming that at least 10 bonds must be detected to register the presence of an analyte, that N=1000 contacts produces 10 detected bonds, A=25,600 μm^2 (equivalent to 64 sensors, each measuring 80 x 5 μm), and t=600 seconds, the minimum detectable concentration of analyte is $1.0 \cdot 10^{-16}$ M, or 65,000 molecules/ml. Thus, even if the active area per probe were significantly smaller than in this example, mass transport limitations would not prevent BARC from equaling or exceeding the ~10^{-12} M sensitivity of other biosensors.

Active area becomes a greater concern when we consider how many beads must be detected and how much area those beads occupy on the substrate. Applications that involve characterizing intermolecular forces (rather than simply detecting an analyte) are the most demanding in this respect, since accurate measurements of intermolecular forces would require observations on at least 100 beads for each analyte. In magnetic bead immunoassays (Fig. 2), we have found that each square millimeter of substrate can accommodate a maximum of 5,000 2.8 μm Dynabeads. If a greater density of beads is deposited, substrate-bound beads attract each other when a magnetic field is applied. This effect prevents the application of known, controlled forces. The active area per analyte must therefore be at least 20,000 μm^2, and a 1 x 1 cm MRAM-type chip would accommodate about 1,000 analytes.

The number of analytes could be increased by using smaller beads such as Sera-Mag Microparticles or Estapor Microspheres (Fig. 4), since more of these beads can be applied per unit area of substrate. The smaller beads also experience significantly greater Brownian motion, so each bead should sample more of the substrate than a Dynabead. Furthermore, preliminary experiments indicate that, by using more sensitive GMR materials, it should still be possible to detect individual beads. On the other hand, it is not so easy to compensate for fact that the smaller beads produce an order of magnitude less magnetic force than Dynabeads. Thus, it is expected that small beads will be optimal for high-sensitivity applications, while large beads will be more useful for measuring intermolecular forces.

CONCLUSION

The concept of measuring intermolecular forces with arrays of magnetoresistive sensors holds considerable promise. The advent of MRAM chips, combined with the growing battery of techniques for generating DNA probes against microbial genomes, could enable a BARC sensor that would be of great use for on-site detection and characterization of pathogenic agents. With its potential ability to make

thousands of ligand-receptor binding affinity measurements in a period of minutes, BARC also has significant potential in the field of drug discovery.

ACKNOWLEDGEMENTS

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FOOTNOTES

1. Using a micromanipulator, we placed 12 different Dynabeads M-280 (lot 3601, expiration date 6/96) at the center of an 80 x 5 μm GMR strip and, as described in this paper, measured the magnetoresistive signal produced by each bead. The standard deviation of the resulting 12 measurements was 72%. When we placed the same bead on the GMR strip 12 times, the standard deviation was only 8%. Thus we estimate that the standard deviation of Dynabead M-280 magnetization is $(72^2 - 8^2)^{1/2} = 72\%$. It has been reported that the standard deviation of Dynabead M-450 magnetization is only 5% [Reddy, S., Moore, L.R., Sun. L. *et al.* (1996) Determination of the magnetic susceptibility of labeled particles by video imaging. *Chem. Eng. Sci.*, **51**, 947–956]; the larger volume of these beads may result in greater magnetic uniformity.
2. This "back-biasing" method has been used by NVE to detect magnetic inks in paper currency, as described on NVE's Internet site, http://www.nve.com/apps/currency/index.html.